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13. ABSTRACT (Maximum 200 Words) <p>The development of human ovarian cancer depends, in part, on formation of an adequate blood supply. Tumor angiogenesis is essential for cancer growth, and vascular endothelial growth factor (VEGF) is critical in stimulating growth of vascular endothelial cells. VEGF is produced by ovarian cancers, and VEGF secretion is markedly higher in ovarian cancers with HER-2 oncogene overexpression. Herceptin, an antibody to HER-2 receptors, has direct antitumor effects, but the antibody also elicits profound reduction in VEGF secretion from ovarian cancer cells, and, thereby, halts tumor-associated angiogenesis. More complete suppression of angiogenesis may be elicited by treatments that synergistically suppress blood vessel proliferation, such as squalamine, an angiostatic steroid designated by the FDA as an orphan drug candidate for therapy of ovarian cancer. Using human ovarian cancer cells in vivo, squalamine elicits antitumor effects by suppressing angiogenic activity of several vascular growth factors including VEGF. This action is due, in part, to squalamine binding at the surface membranes of endothelial cells, leading to blockade of MAP kinase signaling for proliferation. Thus, squalamine shows efficacy alone and combined with other antitumor therapies, including cisplatin, carboplatin and Herceptin, in suppressing the growth of human ovarian cancers with and without overexpression of HER-2 oncogene.</p>				
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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	8
Conclusions.....	9
References.....	9
Appendices.....	

INTRODUCTION

Ovarian cancer is the most deadly gynecologic malignancy. About 26,500 women are diagnosed with this cancer each year and have an overall 5-year survival rate of only 47% (1, 2). For most patients, surgery alone does not cure the cancer due to the spread of tumors beyond the confines of the ovary, and management in the clinic often requires use of toxic chemotherapy regimens. The progressive growth of ovarian cancer depends, in part, on the formation of an adequate blood supply, and tumor angiogenesis has been reported to have prognostic significance in epithelial ovarian cancer (3). Therapy directed toward the vasculature of solid tumors is now being pursued as an important new direction in cancer treatment, because avascular tumors exhibit limited growth (4,5) and tumor aggressiveness and metastatic potential commonly correlate with tumor vascularity (6).

Vascular endothelial growth factor (VEGF) is produced by most solid tumors and elicits a mitogenic effect on tumor-associated endothelial cells (7, 8). VEGF binding to receptor tyrosine kinases triggers activation of downstream signaling enzymes, including p42/p44 MAP kinase, which, in turn, regulate gene expression and specific endothelial cell responses including proliferation, migration, differentiation and apoptosis (9,10). VEGF plays an important role in progression of ovarian cancer (3, 11, 12), and the ability of VEGF to increase vascular permeability (7, 8, 13) may also promote formation of malignant ascites (14). Growth factor pathways, such as those dependent on the HER-2 receptor, appear to up-regulate VEGF production in some solid tumors (15). Since HER-2 receptor is overexpressed in a significant number of ovarian cancers (16, 17), it may also play a role in promoting further growth of ovarian malignancy by increasing VEGF-dependent tumor angiogenesis.

Squalamine, a sterol from tissues of dogfish shark (18), has significant anti-angiogenic and antitumor activity in laboratory models of brain, breast and lung cancer (19-22). Squalamine is a 7,24-dihydroxylated 24-sulfated cholestane steroid conjugated to spermidine at C-3, and it blocks endothelial cell growth and exhibits inhibitory activity in chick embryo chorioallantoic membrane and rabbit corneal micropocket assays (19, 20,23). This anti-angiogenic agent may have good potential for clinical application because it inhibits endothelial cell proliferation induced by a wide range of growth factors, including VEGF (19). This inhibition may result, in part, from its interaction with endothelial cell surface proton pumps, thereby altering intracellular pH and impeding signaling by growth factors (24, 25). When administered as a single agent in nude mice with lung cancer xenografts, squalamine has limited antitumor activity, but the anti-angiogenic steroid significantly enhances the antitumor efficacy of cisplatin and carboplatin/paclitaxel chemotherapies (20-22). Since platinum-based treatments are often used for human ovarian cancers (1, 2), squalamine in combination with cisplatin or carboplatin was studied to assess its utility as part of a coordinated attack against human ovarian cancers and their blood supply. One additional feature we were particularly interested in was the consequence of HER-2 oncogene overexpression for squalamine modulation of growth in ovarian tumor xenografts. Amplification and/or overexpression of HER-2 oncogene in human cancers, including ovarian cancers, is often associated with poor clinical outcome (16,17), and human ovarian tumor cells with overexpression of HER-2 membrane receptor also exhibit resistance to cisplatin and carboplatin (26). We therefore examined whether the level of HER-2 expression in paired HER-2-transfected and non-transfected ovarian cancers influenced the degree of tumor growth inhibition seen with squalamine with or without concomitant platinum-based treatment.

BODY: RESEARCH PROGRESS

AIM 1) Evaluation of angiogenic activity due to HER-2 gene overexpression in human ovarian cells.

1.a. *Squalamine Does not Affect VEGF Secretion in vitro for Ovarian Cancer Cells with or without HER-2 Gene Overexpression.*

HER-2 overexpression is generally thought to lead to tumor development through its effects on promoting uncontrolled cancer cell growth. However, recent findings suggest that HER-2 may also regulate cell survival functions such as angiogenesis by promoting tumor production of VEGF (15). To explore how HER-2 may contribute to angiogenesis in ovarian cancer, we evaluated HER-2 effects on *in vitro* VEGF secretion by human ovarian tumor cells. Parent and HER-2-overexpressing ovarian 2008 cells were incubated for 72 h *in vitro*, and

secretion of VEGF into conditioned media was measured by use of established enzyme-linked immunosorbant assay (see Fig. 6 in ref. 27). Parent ovarian cancer cells show significant secretion of VEGF, and, after transfection of ovarian cells with HER-2 gene to high levels, a further increase in VEGF secretion was found. In parallel *in vitro* studies, treatment of ovarian cancer cells with squalamine elicited no significant effect on secretion of VEGF (27). Thus, HER-2 overexpression may contribute to angiogenesis through up-regulation of VEGF secretion in ovarian cancer, but squalamine is not anti-angiogenic at this step in tumor-associated angiogenesis since it does not appear to directly affect secretion of VEGF by ovarian epithelial tumor cells. This research aim has been completed.

AIM 2) Assessment of biologic activities of squalamine, a newly-synthesized anti-angiogenic steroid, using human vascular endothelial cells *in vitro*.

2.a. Squalamine Blocks VEGF-Stimulated Proliferation of Endothelial Cells *in vitro*.

To assess potential biologic mechanisms for antiangiogenic and antitumor effects of squalamine noted previously, human umbilical vein endothelial cells (HUVEC) were grown *in vitro*. VEGF elicits significant proliferation of HUVEC cells by 72 h. In the absence of VEGF, squalamine has no effect on proliferation or survival of HUVEC cells. However, in the presence of VEGF, squalamine elicited a significant reduction in VEGF-induced endothelial cell proliferation (see Fig. 5 in ref. 27). This growth-suppressive effect of squalamine appears restricted to endothelial cells since the compound had no direct inhibitory effect on the proliferation of ovarian 2008 cancer cells, either with or without HER-2 gene overexpression (27).

This effect of squalamine appears to be due, in part, to binding of squalamine with caveolae fractions purified from plasma membranes of human vascular endothelial cells. An example of purification of a caveolae membrane fraction from human vascular endothelial cells using a detergent-free method is shown in Fig.1. The endothelial cells exhibit enrichment of caveolin-1 in caveolae-related domains. In addition, in 3 experiments, we find localization of specific [³H]-squalamine binding-sites in caveolin-enriched membrane fractions. Further experiments are planned to follow-up on this finding and to evaluate ligand specificity of binding.

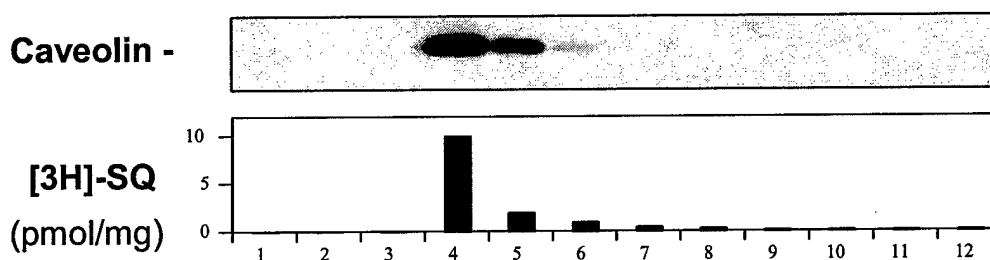


Fig. 1. Purification of caveolae plasma membrane subfractions from human vascular endothelial cells (HUVEC). HUVEC exhibit significant enrichment of caveolin-1 in caveolae membrane domains (see gradient fractions 4-6) isolated from HUVEC cells by use of established detergent-free methods. In addition, specific binding of [³H]-squalamine localizes to those gradient fractions that contain caveolin (fractions 4-6). Specific binding of [³H]-squalamine was assessed by use of established methods. Results from one representative experiment are shown.

The nature of the squalamine binding site and its interaction with other cell signaling components remains to be investigated further. This work may be accomplished within the framework of a no-cost time extension that was requested in an independent communication.

2.b. Squalamine Blocks VEGF-Induced Activation of MAP Kinase *in vitro*.

VEGF exerts its biologic effects by binding with receptor tyrosine kinases, notably Flt-1 and Flk-1/KDR, present at the surface of endothelial cells (9). Post-receptor signal transduction regulates effects of VEGF, and proliferative effects of VEGF in endothelial cells have been associated with VEGF-induced tyrosine phosphor-

ylation and stimulation of mitogen-activated protein kinases (MAP kinase), extracellular signal-regulated kinase ERK-1 (p44^{MAPK}) and ERK-2 (p42^{MAPK}) (9, 10). On the assumption that blockade of endothelial cell proliferation by squalamine may occur, in part, by suppression of MAP kinase signaling cascades induced by growth factors, VEGF-induced tyrosine phosphorylation of MAP kinases was assessed. As expected, VEGF promotes tyrosine phosphorylation of p42/p44 MAP kinase isoforms, with maximal effects evident by 10 min (see Fig. 7 in ref. 27). However, after administration of squalamine, the VEGF-stimulated phosphorylation of MAP kinase isoforms is significantly suppressed, especially after 30 min exposure to VEGF (27). Following success in these studies, additional work will now focus on squalamine-mediated changes in the activity of p38 MAP kinase and the association of p38 MAP kinase with F-actin formation and focal adhesion assembly, important functions in the migration and proliferation of vascular endothelial cells (9,10,25). This latter work may also be accomplished within the framework of a no-cost time extension that was requested in an independent communication.

AIM 3) Investigation of the efficacy of squalamine alone and combined with other antitumor agents in blocking the *in vivo* growth and progression of human ovarian cancer xenografts in nude mice.

3.a. Squalamine and Platinum-Based Chemotherapiess Block Growth of Ovarian Tumor Xenografts in vivo.

Potential antitumor effects of the angiostatic steroid squalamine were assessed in murine tumor xenografts in the absence and presence of cisplatin or carboplatin chemotherapy. Human ovarian 2008 cancer cells without or with HER-2 overexpression were grown as subcutaneous tumors in nude mice. Tumors were grown to 150-200 mm³ in size. Then, animals with established tumors were treated with control solution, cisplatin alone at two different dose levels (4 mg/kg on day 1 or 5 mg/kg on days 1,8), squalamine alone (2 mg/kg) on days 1-10, or cisplatin in combination with squalamine (days 1-10) (see Fig. 1 in ref. 27). In one set of experiments, animals were treated with a high dose of cisplatin near the maximum tolerated dose (5 mg/kg on days 1,8) (27). In the second set of experiments, lower doses of cisplatin that resulted in only partial growth inhibition (26,29) were chosen in order to ensure use of the chemotherapeutic agent at a level that would not totally suppress tumor growth, thus allowing detection of any potential additive effects of a squalamine-cisplatin interaction. By 28 days, both 2008 parental and HER-2-overexpressing tumors showed little overall response to therapy with the lower dose of cisplatin alone. However, 2008 parental and HER-2-overexpressing tumors exhibited some minor response to cisplatin alone administered at the higher dose level (27). Squalamine elicited a partial reduction in tumor size as compared to controls in both 2008 parental and HER-2-overexpressing tumors. More profound tumor growth inhibition was elicited by combined treatment with squalamine and cisplatin in both 2008 parental and HER-2-overexpressing cancers (27). This effect of combination therapy was found when squalamine was administered with either low or high doses of cisplatin (27).

The antitumor effects of squalamine with and without platinum-based chemotherapy were also assessed using a different human ovarian tumor xenograft, CAOV3, that has been transfected to exhibit HER-2 overexpression (see Fig. 2A in ref. 27). After tumor growth to 50-60 mm³, animals were treated with control solution, carboplatin alone (60 mg/kg) on day 1, squalamine alone (2 mg/kg) on days 1-10, or carboplatin (day 1) in combination with squalamine (days 1-10). By 28 days, CAOV3 HER-2-overexpressing tumors showed minimal response to therapy with carboplatin alone (27). As with the 2008 tumors, squalamine as a single agent elicited a partial reduction in CAOV3 tumor size as compared to controls (27). More marked inhibition of tumor growth was elicited by combined treatment with squalamine and carboplatin (27).

The tumor growth inhibition seen with combined squalamine and platinum-based chemotherapeutics for both human ovarian tumor lines persisted for up to 18 days following cessation of squalamine treatment. We therefore investigated how long bioactivity persisted with combined cisplatin and squalamine treatment of HER-2-overexpressing CAOV3 tumors by maintaining the dual therapy animal cohort until the mean tumor size for these animals reached 500 mm³ (see Fig. 2B in ref. 27). After tumor growth to 50-60 mm³, animals with established tumors were treated with control solution, cisplatin alone (4 mg/kg) on day 1, squalamine alone (2 mg/kg) on days 1-10, or cisplatin (day 1) combined with squalamine (days 1-10). As compared with control tumor xenografts, the calculated tumor growth delay in established tumors was 7 days for cisplatin therapy

alone, 28 days for squalamine treatment alone, and 91 days for squalamine with cisplatin (27). Combined squalamine-cisplatin therapy was nontoxic as assayed by no animal death or significant weight loss during the study period (27). This aim has been completed.

3.b. *Squalamine and Cisplatin Promote Ovarian Tumor Cell Apoptosis in vivo.*

To assess molecular effects of squalamine and cisplatin, ovarian 2008 parent and HER-2-overexpressing tumor xenografts remaining after treatments with squalamine, cisplatin or a combination of the reagents were harvested and assessed for ovarian tumor cell apoptosis *in vivo*. For evaluation of apoptosis, the modified TUNEL assay (34, 35) was performed on tissue sections. The assays showed evidence of increased apoptosis in ovarian 2008 parental tumor cells treated with squalamine alone, cisplatin or combined cisplatin-squalamine as compared to appropriate controls (all at $P < 0.05$) (see Fig. 3A in ref. 27). The 2008 HER-2-overexpressing ovarian tumors displayed less apoptotic activity than 2008 parental cancers with all treatments (27). Although apoptosis tended to be higher after administration of either squalamine or cisplatin alone, only treatment with squalamine in combination with cisplatin elicited a significant increase in the extent of apoptosis of HER-2-overexpressing ovarian cancers ($P < 0.001$) (27). The results suggest that squalamine enhances cytotoxic effects of cisplatin chemotherapy for human ovarian cancer cells by increasing levels of tumor cell apoptosis produced by cisplatin exposure, either with or without HER-2 oncogene overexpression. In independent experiments, similar results have been obtained after treatments with squalamine in combination with carboplatin (data not shown). This aim has been completed.

3.c. *Squalamine Down-Regulates Ovarian Tumor-Associated Angiogenesis but not VEGF Production in vivo.*

Tissue sections of parent and HER-2-overexpressing 2008 tumor xenografts remaining after treatments with squalamine, cisplatin or a combination of the reagents were prepared for immunohistochemical staining with human von Willebrand Factor (vWF) to detect blood vessels (27,34). On scoring of tumor microvessel density, 2008 HER-2-overexpressing tumors exhibited more angiogenic activity than 2008 parental cancers ($P < 0.001$) (see Fig. 3B in ref. 27). Treatment with squalamine alone elicited a reduction of tumor-associated blood vessel density for either ovarian tumor (27), and the immunohistochemical analyses also revealed a reduction of tumor-associated angiogenesis in mice treated with cisplatin plus squalamine ($P < 0.01$) (27). No significant differences in microvessel density were found between groups treated with cisplatin alone and controls. The results suggest that squalamine is antiangiogenic for ovarian cancer cells with or without HER-2 overexpression. Squalamine-induced suppression of tumor microvessels is also a sustainable event since it was noted up to 18 days following the last squalamine dose. Similar studies based on treatments with squalamine combined with carboplatin are confirmatory. This aim has been completed.

KEY RESEARCH ACCOMPLISHMENTS

- Profound growth inhibition was elicited by squalamine alone and by combined treatment with squalamine and cisplatin or squalamine and carboplatin for both parental and HER-2-overexpressing ovarian tumor xenografts.
- Immunohistochemical evaluation of tumors revealed decreased microvessel density and increased apoptosis. Although HER-2-overexpressing tumors had more angiogenic and less apoptotic activity than parental cancers, growth of both tumor types appear to be similarly suppressed by treatment with squalamine combined with cisplatin or squalamine combined with carboplatin.
- In *in vitro* studies, we found that squalamine does not directly affect proliferation of ovarian cells. However, squalamine significantly blocked VEGF-induced activation of p42/p44 MAP kinase and cell proliferation in human vascular endothelial cells.

- Squalamine binds with high avidity to a caveolin-enriched plasma membrane domain purified from human vascular endothelial cells. This membrane fraction corresponds with caveolae, a region that functions as a 'signaling platform' for the regulation of vascular cell growth.
- The results suggest that squalamine is anti-angiogenic for ovarian cancer xenografts and appears to enhance cytotoxic effects of cisplatin and carboplatin chemotherapy independent of HER-2 tumor status.
- It is important to note that this preclinical work has helped to promote the initiation of independent clinical trials of squalamine for treatment of patients with resistant or recurrent ovarian cancer (38).

REPORTABLE OUTCOMES

Presentations

1. Pietras, R.J. "Squalamine-induced blockade of tumor-associated angiogenesis in human ovarian cancers". Presented at Jonsson Comprehensive Cancer Center Lecture Series, UCLA (February, 2002).
2. Pietras, R.J. "New approaches to antitumor therapy". Presented at Marion Medical Center Cancer Forum, Santa Maria, California (September, 2002).
3. Pietras, R.J. "Squalamine blocks growth and tumor-associated angiogenesis of human ovarian cancer". To be presented at XVII FIGO World Congress of Gynecology and Obstetrics, Santiago, Chile (November, 2003).
4. Pietras, R.J. and Marquez D. "Steroidal agents and tumor-associated angiogenesis". To be presented at FASEB Summer Research Conference, Tucson, Arizona (July, 2004).

Publications

1. Li, D., Williams, J. I. and Pietras, R.J. (2002). Squalamine and cisplatin block angiogenesis and growth of human ovarian cancer cells with or without HER-2 gene overexpression. *Oncogene* 21 : 2805-2814.
2. Pietras, R.J., Gorrin-Rivas M. and Chen, H.-W. (2003). Squalamine blocks tumor-associated angiogenesis and growth of ovarian cancer. *Proc. FIGO World Congress Gynecol Obstet* 17 (in press).

Additional Research Opportunities

Results from the preclinical research activity outlined above has led to the promotion of new clinical trials of squalamine in the treatment of patients with resistant or recurrent ovarian cancer. The PI of the present grant, Dr. Pietras, was a co-investigator in this ongoing series of clinical trials:

1. Davidson, S. A., Chap, L., Pietras, R., Astrow, A., Gajewski, W., Brader, F., Petrone, M., Desai, A., Solomon, S., Holroyd, K., Major, F., Adler, L. and Cohn, A. (2002). A Phase IIA trial of continuous 5-day infusions of MSI-1256F (squalamine lactate) plus carboplatin for therapy of persistent or recurrent advanced ovarian cancer. *Proc. Am. Soc. Clin. Oncol.* : 878.

No patents, development of cell lines, informatics or additional funding opportunities to be reported at this time.

CONCLUSIONS

The potential role of squalamine, a natural anti-angiogenic steroidal compound, in treatment of ovarian cancers with or without standard cisplatin or carboplatin chemotherapy was assessed. Since HER-2 gene overexpression is associated with cisplatin and carboplatin resistance *in vitro* and promotion of tumor angiogenesis *in vivo*, the response of ovarian cancer cells with or without HER-2 gene overexpression to squalamine and cisplatin or squalamine and carboplatin was also evaluated in tumor xenograft models and in tissue culture. Profound growth inhibition was elicited by squalamine alone and by combined treatment with squalamine and cisplatin or squalamine and carboplatin for both parental and HER-2-overexpressing ovarian tumor xenografts. Immunohistochemical evaluation of tumors showed decreased microvessel density and increased apoptosis. Although HER-2-overexpressing tumors had more angiogenic and less apoptotic activity than parental cancers, growth of both tumor types was similarly suppressed by treatment with squalamine combined with cisplatin. In *in vitro* studies, we found that squalamine does not directly affect proliferation of ovarian cells. However, squalamine binds with high avidity to vascular endothelial cell caveolae, plasma membrane subdomains that function as 'signaling platforms' for the regulation of vascular endothelial cell growth. As a consequence of primary interactions at the plasma membrane, squalamine significantly blocks VEGF-induced activation of p24/p44 MAP kinase and cell proliferation in human vascular endothelial cells. The results suggest that squalamine is anti-angiogenic for ovarian cancer xenografts and appears to enhance cytotoxic effects of cisplatin and carboplatin chemotherapy independent of HER-2 tumor status. In addition, it is important to note that this preclinical work has helped to promote the initiation of Phase II clinical trials of squalamine for treatment of patients with resistant or recurrent ovarian cancer. Further experiments are continuing in our laboratory in accord with our statement of work, and a no-cost time extension has been requested to facilitate completion of this important research.

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